Plant Physiol. (1984) 74, 675–680
0032-0889/84/74/0675/06/$01.00/0

A Seed Storage Protein with Possible Self-Affinity through Lectin-Like Binding[1]

Received for publication July 12, 1983 and in revised form September 27, 1983

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ABSTRACT

The primary storage protein of oat (*Avena sativa* L.) seeds, globulin, was shown to have a specific carbohydrate-binding activity. The globulin was capable of hemagglutinating rabbit red blood cells and this hemagglutination was inhibited by the β-glucan, laminarin, as well as by carbohydrate which had been cleaved from the native globulin. Globulin with carbohydrate-binding activity was isolated from cell wall preparations and from defatted flour. The lectin activity apparently resides in the α-subunit of the globulin and has affinity for the carbohydrate which is *O*-glycosidically linked to the globulin. A portion of this carbohydrate is attached to the β-subunit. Two affinity columns were synthesized utilizing laminarin and the carbohydrate from the native globulin as ligands. The hemagglutinating activity bound to both of these columns. The activity was specifically eluted from the globulin-carbohydrate affinity column with carbohydrate cleaved from native globulin by an alkali-catalyzed β-elimination. The possible roles of this unique self-binding capacity are discussed.

Two principal classes of proteins that dominate the protein content of many plant seeds are the storage proteins and the lectins. In agronomically important cereals, examples of both classes may be found; however, in oats (*Avena sativa* L.), only the storage proteins have been reported (7). Oat globulin, the salt-soluble protein fraction, is the primary storage protein in oat seeds, and constitutes as much as 75% of the total seed protein. This multimeric protein has been reported to be composed of six dimers with a total mol wt of approximately 348,000 (3). Interchain disulfide bonds were demonstrated *in vivo*, forming heterogeneous dimers of α and β polypeptides. The α polypeptides range in mol wt from 32,500 to 37,500 and the β polypeptides range in mol wt from 22,000 to 24,000 (5). The isoelectric pH values range from 5.9 to 7.2 for the several (≥6) acidic α subunits and from 8.7 to 9.2 for the several (≥5) basic β subunits (5). The high degree of heterogeneity of the oat globulin is similar to the heterogeneity of seed storage proteins of other species (8, 26, 28) which has been attributed to the expression of multigene families (6). Further characterization of the oat globulin includes amino acid composition (5) and evidence for the synthesis of higher mol wt precursors (6).

Lectins, or carbohydrate-binding proteins, have been reported to comprise substantial quantities of seed protein, especially in legumes. Although lectins have been described from several cereals, no lectin has previously been reported in oats (21). Despite many years of exhaustive investigation, no physiologic function has yet been conclusively proven for these intriguing proteins. Proposed functions include: determinants of host/symbiont specificity (4); plant defense mechanisms (20); sugar transport or glycosidase enzymes (6); storage proteins (6); and embryological functions in cell-cell interactions (1).

We present data that purified oat globulin binds specific carbohydrates and causes hemagglutination of rabbit red blood cells. Furthermore, the lectin activity can be inhibited by carbohydrates derived from the oat globulin itself. An affinity resin was synthesized for the globulin using the carbohydrate-containing tryptic peptides from the globulin as the ligand. This possible specific self-recognition and self-association capability could provide either an efficient packing mechanism of the native globulin multimer or participate in the association of newly synthesized globulin subunits.

MATERIALS AND METHODS

Purification of the Lectin from Cell Walls. Oat cell walls were prepared by the method of Mares and Stone (19) from the cultivar Froker (*Avena sativa* L.). Cell walls (60 g) were stirred overnight in 1% SDS, 50 mM Tris-HCl, pH 7.0 (25°C), to remove any globulin which may have become associated with them during the cell fractionation procedure and the mixture was centrifuged (10,000*g* × 20 min, 4°C). The resulting pellet was washed with distilled water and ground in 80 ml of 50 mM Tris, pH 7.0 buffer, with a mortar and pestle. The solution was stirred for 45 min and then reground. The mixture was centrifuged (10,000*g* × 20 min) and the supernatant was lyophilized and dissolved in 10 ml of 50 mM phosphate, pH 6.5, and centrifuged to clarify. The sample was dialyzed against 50 mM phosphate, pH 6.5.

The sample was adsorbed onto a column of Whatman DEAE-cellulose (DE52)[2] (2 × 30 cm) previously equilibrated in 50 mM phosphate, pH 6.5. The lectin was eluted with a linear gradient of KCl (0–0.3 M) in the same buffer. The fractions containing the lectin activity were pooled and concentrated by lyophilization. The sample (1.2 mg, 512 HAU[3]/mg) was dialyzed against 50 mM phosphate, pH 8.5.

Purification of Globulin from Defatted Oat Flour. The method of Brinegar and Peterson (5) was used to purify the oat globulin

[1] Cooperative investigation of the United States Department of Agriculture, Agricultural Research Service, and the College of Agricultural and Life Science, University of Wisconsin-Madison.

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[3] Abbreviation: HAU, hemagglutination unit.

from defatted oat flour from the cultivar Froker (*Avena sativa* L.). The flour was stirred with 50 mM Tris-HCl, pH 8.0 (25 ml/g flour) for 45 min at room temperature to remove the albumins. After chilling, the extract was centrifuged (27,000*g* × 15 min at 4°C). The supernatant was discarded and the residue was stirred at room temperature with 1 M NaCl, 50 mM Tris-HCl, pH 8.0 (25 ml/g flour). The mixture was centrifuged (27,000*g* × 15 min at 20°C) and the supernatant was extensively dialyzed against distilled water at 4°C. The precipitated globulin was recovered by centrifugation at 4°C and lyophilized.

Alkali-Catalyzed β-Elimination of Globulin Carbohydrate. Oat globulin (200 mg) prepared by the method of Brinegar and Peterson (5) was dissolved in 20 ml of 0.3 M NaOH containing 12.5 mCi of NaB^3H_4 and 0.3 M $NaBH_4$ and the reaction was allowed to proceed for 72 h. The rate of the reaction was monitored by observing the change in *A* at 241 nm (24) (shorter reaction times resulted in incomplete cleavage and reduced yields). The pH was brought to 3 with 3 N acetic acid and the solution was stirred until hydrogen evolution ceased. The solution was then neutralized with 1 N NH_4OH. The precipitated protein was removed by centrifugation (10,000*g* × 15 min) and the supernatant which contained the carbohydrate was retained. Borate was removed as methylborate by the following procedure. The interfering cations were first removed by passing the sample through a column of Dowex AG-50. This step was followed by repeated reaction (4×) with excess methanol at pH 7.5 (titrated with NH_4OH). The volatile methylborate, methanol, and NH_4OH were removed by rotatory evaporation at reduced pressure. The sample (2.0 mg carbohydrate) was applied to a column of Bio-Gel P-2 (1.2 × 90 cm) equilibrated in 0.1 M $(NH_4)_2CO_3$, pH 7.0, and the sample was eluted with this same buffer. A typical yield of 2 to 2.4 mg of carbohydrate was obtained. The carbohydrates resulting from the β-elimination reaction were identified by their tritium content as monitored by liquid scintillation.

Preparation of Two Affinity Columns. An affinity column (30-ml bed volume) was prepared by the method of Uy and Wold (30) using β-laminarin as the ligand. Laminarin (Sigma), 150 mg in 19 ml of 0.1 N NaOH, was used with 30 g of Sepharose 6B in the coupling procedure.

A second affinity column was prepared from the globulin carbohydrate. Pure oat globulin (from defatted oat flour) (0.41 mg/ml, 95 ml) was digested with trypsin (1 mg/ml) in a solution of 1 M $CaCl_2$ for 30 min at room temperature. The reaction was stopped by heating (70°C for 10 min), the solution was lyophilized, and the peptide fragments were solubilized in 8 M urea (7 ml). The sample was chromatographed on a column of Bio-Gel P-10 equilibrated in 6 M urea, 50 mM Tris-HCl, pH 8.0 buffer, and the fractions containing both protein and carbohydrate were pooled. SDS-polyacrylamide gel electrophoresis of an aliquot of the trypsin digest showed that only protein fragments of less than M_r = 5000 remained. Those peptide fragments with the carbohydrate attached were then coupled to CNBr-activated Sepharose 4B by the following procedures. An aliquot of these fragments (5 mg protein, 2 mg carbohydrate) obtained from gel filtration was extensively dialyzed (Spectra/Por 1000 MWCO) against the buffer for the coupling reaction (0.1 M $NaHCO_3$, 1 M NaCl, pH 8.3). A precipitate of insoluble peptide fragments was removed by centrifugation (10,000*g* × 10 min). The supernatant which contained 1.4 mg protein and 2 mg carbohydrate was combined with CNBr-activated Sepharose 4B (1 g) and allowed to react for 2 h with frequent agitation. The gel was allowed to settle and the supernatant was removed by decantating. Ten ml of 0.2 M glycine, pH 8.0, was added and allowed to react at 4°C overnight with agitation. The gel was allowed to settle and the supernatant was removed by decanting. The gel was poured into a column (3.5-ml bed volume) and washed with 50 ml of 0.1 M acetate, 0.5 M NaCl, pH 4. This was followed by washing with 50 ml of the coupling buffer.

Deglycosylation of Globulin. The deglycosylation of the globulin prepared by the procedure of Brinegar and Peterson (5) was carried out by the procedure of Edge *et al.* (10) using trifluoromethane-sulfonic acid. A sample size of 10 mg was used, and the protein was freed of reagents and low molecular weight sugars by the ether precipitation step described in their procedure.

Hemagglutination Assay. The microhemagglutination assay was employed using serial 2-fold dilutions (50 μl) plus 150 μl of trypsinized and glutaraldehyde-treated (29) rabbit red blood cells. Hemagglutinating activity was determined after 1 h and the activity was recorded in hemagglutination units defined as the reciprocal of the greatest dilution of 50 μl of lectin solution giving observable agglutination (11). The activities are expressed as a specific hemagglutinating activity based on the use of a 1 mg/ml protein solution in the initial lectin solution.

Other Procedures. The sugars were analyzed by descending paper chromatography (Whatman 3 MM) for 18 to 22 h using butanol, pyridine, and H_2O in a ratio of 6:4:3. The sugars were visualized after treating the chromatogram with alkaline silver nitrate (27). Laminarin (Sigma) (2.0 mg) was fractionated into high and low molecular weight species by chromatography on a column of Bio-Gel P-100 (1.4 × 92 cm). The low molecular weight fractions (less than M_r = 10,000) were more effective at inhibiting the hemagglutination reaction than was the unresolved mixture of high and low molecular weight polysaccharides. Carbohydrate was determined by the phenol-sulfuric acid assay (9). Protein was measured by the method of Lowry *et al.* (18). Gel electrophoresis and isoelectric focusing procedures were those of Brinegar and Peterson (5). The gels were stained for protein with Coomassie Blue. Ampholytes of the pH range 3 to 10 were purchased from LKB Produkter.

RESULTS

Identification of the Purified Globulin as a Lectin. Purified native globulin isolated by the method of Brinegar and Peterson (5) was capable of hemagglutinating rabbit red blood cells with an activity of 32 HAU/mg. A hemagglutinating activity of 512 HAU/mg was isolated from the cell wall fraction. This preparation was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The subunit mol wt (35,000 and 23,000) of the major polypeptides of the cell wall fractions containing the hemagglutinating activity were found to be the same as those of native globulins. More than 80% of the protein in these samples was found to be globulin. The hemagglutinating activities of both the native globulin preparation and of the cell wall fraction were completely inhibited (>99%) by laminarin of M_r = 5000–10,000 (300 μg/ml); but not as effectively by larger laminarin (M_r > 20,000). Carbohydrate cleaved from the globulin by the base-catalyzed β-elimination (150 μg/ml) was also an effective inhibitor of the agglutination assay. This observation was considered more interesting since it may represent a physiologically important ligand. In addition to the carbohydrate reversibility, the hemagglutination activity was shown to be the result of a lectin-like protein utilizing the following standard controls for characterizing lectin activity. The hemagglutination was completely destroyed by either heating the lectin at 95°C for 10 min or by treatment of the protein preparation with pronase (10:1, lectin:pronase; 1 h, 37°C). The hemagglutination was not affected by treatment of the lectin with RNase. The hemagglutination activity was not inhibited by 0.1 M concentrations of any of the common hexoses (glucose, mannose, galactose, talose, or glucosamine) or pentoses (ribose, arabinose, or xylose), lactose, maltose, raffinose, stachyose, or sucrose, or by chitin hydrolysates, dextrans of M_r < 10,000 (Sigma), or galactans.

The reduced specific hemagglutinating activity of the globulin

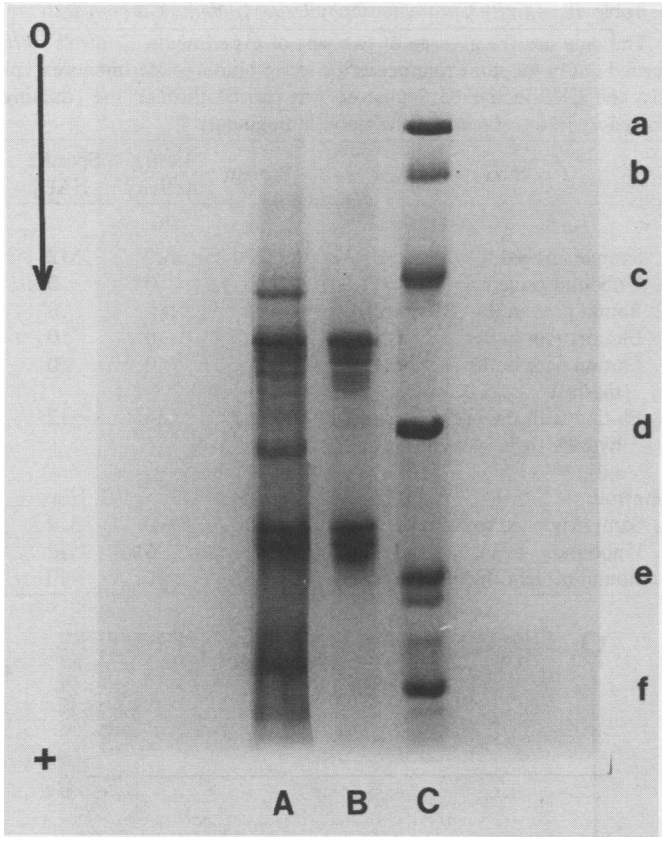

FIG. 1. Globulin analyzed by SDS-polyacrylamide gel electrophoresis. Samples of (A) partially purified globulin from the cell wall fraction (20 μg) and (B) purified globulin (10 μg) were analyzed. The (C) mol wt standards used were: a, phosphorylase , *b*, M_r = 92,500; b, bovine serum albumin, M_r = 66,000; c, ovalbumin, M_r = 45,000; d, carbonic anhydrase, M_r = 31,000; e, soybean trypsin inhibitor, M_r = 21,500; f, lysozyme, M_r = 14,400.

from protein bodies (5) as compared with the cell wall preparation was attributed to loss of activity due to the severity of the purification procedure which utilizes extensive dialysis against distilled H_2O and precipitation of the globulin (5). This observation is consistent with the failure of repeated attempts to completely restore activity to lectin purified from the cell walls which had precipitated during storage. This finding is also consistent with those of Paulova *et al.* (22) who report incomplete restoration of activity for the lectins of lentils, wax beans, lima beans, or soybeans after precipitation of these lectins.

The α and β subunits of the globulin prepared and separated by the method of Brinegar and Peterson (5) were tested for hemagglutinating activity. β subunits did not cause agglutination. The α subunits hemagglutinated the red blood cells (32 HAU/mg), indicating some type of multivalency of carbohydrate binding.

Most lectin subunits contain only a single sugar-binding site and thus must exist as multimers in order to be detected by the hemagglutination assay. It is likely that the α subunits of the globulin also contain a single sugar-binding site and in order to be capable of any hemagglutination must associate with each other to provide multivalent complexes. The α subunits have been shown to aggregate at the low ionic strength of the microtiter assay (5). The reduced titer observed for these subunits may well be due to the failure of the α subunit to associate into complexes capable of efficient hemagglutination. The reduced titer may also attributed to changes in sugar-binding ability after the α and β subunits had been separated, a procedure which included their preparation as the carboxyamidomethylated peptides.

Characterization of Globulin as a Glycoprotein. The globulin (prepared by the method of Brinegar and Peterson [5]) contained 3% by weight of carbohydrate as measured by the phenol-sulfuric acid method (9). Approximately 40% of this carbohydrate was removed by treatment with alkali which catalyzed a β-elimination reaction cleaving the *O*-glycosidic linkages between the carbohydrate and either serinyl or threonyl residues. The reaction proceeded with a concomitant increase in *A* at 241 nm characteristic of the anhydroamino acids, α-aminoacrylic acid and α-aminocrotonic acid (24). Chromatography of the alkali-released carbohydrate on a column of Bio-Gel P-2 resolved two mol wt species. The small saccharide had the same mobility during paper chromatography as maltotriose and was thus estimated to contain 3 to 4 hexose units. The larger saccharide eluted at 4.8 ml after the void volume of the Bio-Gel P-2 column and was estimated to contain 7 to 10 hexose units. The mixture of the two saccharides was hydrolyzed by treatment with 2 N HCl for 2 h at 37°C and the product was analyzed by paper chromatography. The mobility of the major component (>95% of the radioactivity), as detected by liquid scintillation, was coincident with the mobility of D-glucitol, indicating that glucose is *O*-glycosidically linked to either the serinyl or the threonyl residues of the protein. Silver nitrate was used to visualize the alditols resulting from hydrolysis and only glucitol was detected. The $NaBH_4$ was used at a high concentration (0.3 M) to minimize any possible successive hydroysis (peeling) of the polysaccharide during the alkali-catalyzed β-elimination but our results do not absolutely rule out the possibility that at least some peeling did occur.

Deglycosylation of the globulin with trifluoromethane-sulfonic acid is a procedure which also cleaves *O*-glycosidic linkages but does not alter the protein (10). Treatment with this reagent removed 43% of the total carbohydrate. The remaining 60% of the attached carbohydrate was presumed to be attached through an *N*-glycosidic linkage. Globulin deglycosylated by treatment with trifluoromethane-sulfonic acid was compared with pure native globulin by isoelectric focusing (Fig. 2). Pure globulin analyzed by isoelectric focusing appeared to be the same as that analyzed by Brinegar and Peterson (5). Removing the carbohydrate altered the isoelectric pH values of only the basic polypeptides which were shifted to higher pH values. This result suggests that acidic sugars were removed from these polypeptides and that at least a portion of the removed carbohydrate had been attached to the basic β subunits.

Carbohydrate Binding Specificity: Possible Self-binding. High concentrations (≥5 mg/ml) of active lectin (512 HAU/ml) did not hemagglutinate the red blood cells until the sample had been titered using 3 to 4 wells; further dilutions of the material caused positive agglutination until the end-point was reached at concentrations of 2 to 4 μg/ml. This result suggested that high concentrations of the lectin inhibited or interfered with the hemagglutination process. This is consistent with the finding that the hemagglutination was inhibited by the carbohydrate removed from the globulin by base-catalyzed hydrolysis (150 μg/ml). Two affinity columns were synthesized using (a) β-laminarin (Table I) and (b) the carbohydrate-containing portion of a tryptic digest of native globulin (Table II) as the ligands to further test the apparent self-affinity of this globulin.

Affinity Column I. Lectin activity was extracted from cell walls as described. The anion exchange chromatography step was omitted. The sample was dialyzed against 10 mM phosphate buffer, pH 9.0. An aliquot (100 mg, 8 HAU/mg) was applied to the laminarin-affinity column (1.5 × 17 cm) which had been equilibrated in the same buffer. No lectin activity was detected in the effluent. Protein (95.5 mg) eluted during the application of the sample and the subsequent washing of the column in the

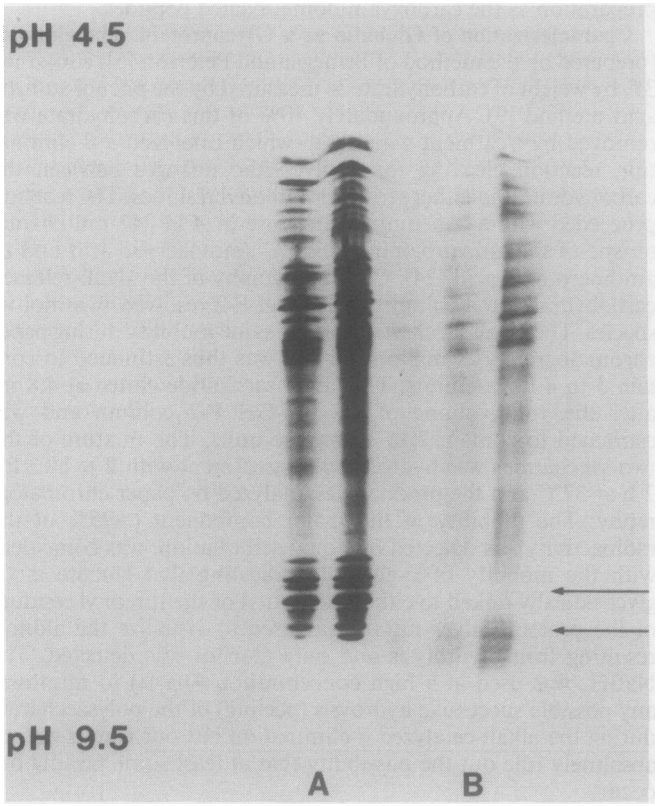

FIG. 2. Analysis of native and deglycosylated globulin subunits. Samples of (A) native (25 and 50 μg) and (B) deglycosylated globulin were analyzed by isoelectric focusing in 6 M urea. Arrows indicate the isoelectric pH of the basic subunits (pI 8.7 to 9.2) of native and deglycosylated globulin. The isoelectric pH of the acidic subunits (pI 5.9 to 7.2) were not altered by treatment with trifluromethane-sulfonic acid. Proteins were stained with Coomassie Blue. Samples were prepared for analyses by isoelectric focusing by first treating them with 1 mM dithiothreiotol. The isoelectric focusing was carried out using a vertical slab (0.77 mm thick) containing 6 M urea and 2% Ampholines (pH 3–10).

Table I. *Affinity Chromatography Using β-Laminarin*

The data are the average of two sets of experiments. Controls were carried out in the same manner as the lectin-binding experiments except that the activated Sepharose was carried through the coupling procedure with glucose instead of the laminarin.

Step	Protein	Lectin Activity	Specific HAU
	mg	*%*	
1. Sample applied to column	100	100	8
2. Unbound protein	95.5	0	
3. Bound protein (by difference)	4.5	100	
4. Elution with buffer	0	0	
5. Elution with buffer and ethylene glycol (5%)	1.2	>95	512
Controls			
1. Sample applied to column	110	100	8
2. Unbound protein	110	100	8
3. Bound protein (by difference)	0	0	

same buffer. The column was washed with 10 mM phosphate buffer, pH 9.0, containing 5% ethylene glycol. Hemagglutinating activity (1.2 mg, 512 HAU/mg) eluted in the first 28 ml after the void volume (10 ml). These fractions were pooled, concentrated by lyophilization, and dialyzed against 10 mM phosphate buffer, pH 8.5. The yield was ≥95% of the total activity applied to the column. The specific hemagglutinating activity was increased 63-fold. The activity was inhibited by the carbohydrate released from the globulin (150 μg/ml). The sample was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Hemagglutinating activity did not bind to Sepharose 6B treated as above

Table II. *Affinity Chromatography Using Globulin Carbohydrates*

The data are the average of two sets of experiments. Controls were carried out in the same manner as the lectin-binding experiments except that the CNBr-activated Sepharose was carried through the coupling procedure in the absence of the globulin fragments.

Step	Protein	Lectin Activity	Specific HAU
	mg	*%*	
1. Sample applied to column	0.200	100	512
2. Unbound protein	0.06	0	0
3. Bound protein (by difference)	0.14	100	0
4. Elution with buffer	0	0	0
5. Elution with buffer + sucrose (2 mg/ml)	0	0	0
6. Elution with the buffer + carbohydrate from globulin (1 mg/ml)	0.075	54	512
Controls			
1. Sample applied to column	0.11	100	512
2. Unbound protein	0.1	90	512
3. Bound protein (by difference)	0.01	10	0

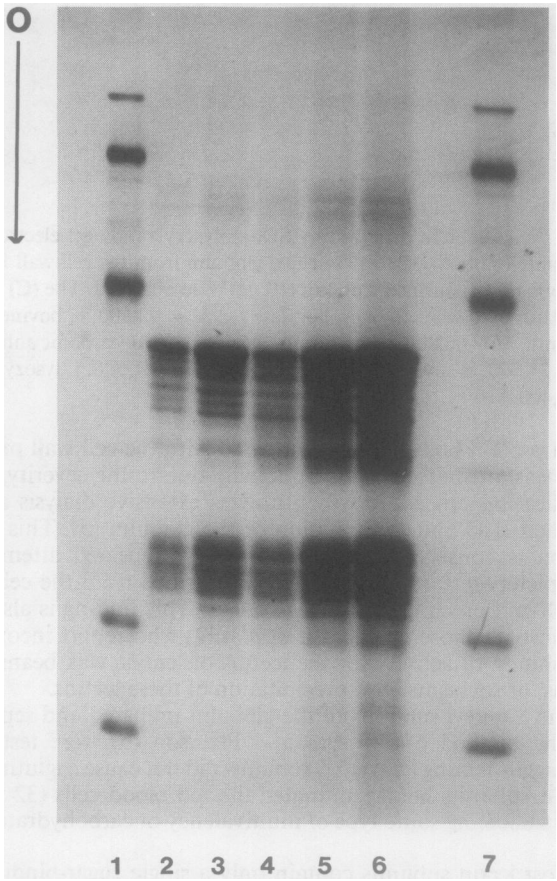

FIG. 3. Hemagglutinating protein released from β-laminarin affinity column analyzed by SDS-polyacrylamide gel electrophoresis. 2 and 3, purified oat globulin (10 and 15 μg). 4, 5, and 6, protein eluted with ethylene glycol (5, 10, and 15 μg) The mol wt standards (1 and 7) are the same as used in Figure 1.

with glucose used to block the reactive groups.

Affinity Column II. The column to which globulin carbohydrate was coupled was equilibrated in 50 mM phosphate, pH 6.5. A sample containing hemagglutinating activity extracted from the cell walls (0.200 mg, 512 HAU/mg) was applied to the affinity column. The sample contained greater than 80% globulin (Fig. 1). The sample was maintained as a dilute solution (0.200 mg/15 ml) and the pH was adjusted from 8.5 to 6.5 immediately before applying the sample to the column at a flow rate of 5 ml/h. The column was washed with 50 mM phosphate, pH 6.5. No hemagglutinating activity was detected in the pooled, lyophilized column effluent which contained 30% of the protein applied to the column (0.060 mg). A solution of carbohydrate released from alkali-treated native globulin was prepared at a concentration of approximately 1500-fold molar excess of free carbohydrate to bound globulin subunits (1 mg/ml, 2 ml) and was allowed to flow into the column. The buffer flow was stopped and the lectin was allowed to equilibrate between binding of free and attached carbohydrate (1 h). The column was washed with 50 mM phosphate, pH 6.5. The first three void volumes (3 ml) were collected and pooled. The pool was concentrated by lyophilization and dialyzed against 50 mM phosphate, pH 8.5 (1:1000 for 14 h) to remove the carbohydrates. The concentrated sample was found to contain approximately 50% of the hemagglutinating activity originally applied to the column (0.075 mg protein, 512 HAU/mg). The specific activity of the material eluted from the column (512 HAU/mg) was the same as that of the sample applied to the column. This result indicates that the hemagglutinating activity is not a result of a minor protein contaminating the sample. This activity was inhibited by β-laminarin (300 μg/ml). The inability to release all of the unbound protein with the carbohydrate suggests that the equilibrium between occupied and unoccupied carbohydrate-binding sites lies in favor of the occupied state at this pH. This interpretation is consistent with our observation that samples solubilized in buffer of pH 8.5 and then assayed by the microtiter assay at pH 6.5 gave greater titer values (512 HAU/mg) than samples solubilized in buffer of pH 6.5 and then assayed (128 HAU/mg).

Controls. (a) Attempts to elute the hemagglutinating activity with sucrose (2 mg/ml) or buffer with no added carbohydrate failed. (b) CNBr-activated Sepharose was carried through the coupling procedure without the trypsin digest material but with glycine added to block the reactive groups as in the original procedure. Greater than 90% of the hemagglutinating activity eluted during sample application to a column of this derivatized sepharose.

Solubility-Stability. According to the classical definition of a globulin, a salt-extractable storage protein, the lectin is a globulin from the oat seed. It was most soluble above pH 8.5 and in at least 0.5 M NaCl; it was much less soluble at pH 7.0. The lectin could not be stored in solution for longer than 1 week, at which time substantial precipitation of the protein began to occur. No activity could be recovered after resolubilization of the precipitated lectin.

DISCUSSION

The carbohydrate-binding capability of the globulin satisfies the basic tests applied to classify such proteins as lectins. However, this protein is less effective at hemagglutinating red blood cells when compared with the high sugar-binding affinity of such lectins as soybean concanavalin A (11). These activities are based on binding affinity for sugars on the cell membrane of rabbit red blood cells which are not necessarily good substrates for carbohydrate-binding proteins of plant origin (7). The globulin is a very heterogeneous protein composed of nonidentical acidic and basic subunits. At least six species of acidic (α) subunits and four species of basic (β) subunits were found by Brinegar and Peterson (5). These groups of acidic (α) and basic (β) subunits apparently randomly associate to form heterodimers which further associate to produce globulin of extremely great heterogeneity as judged by SDS-polyacrylamide gel electrophoresis and by isoelectric focusing (Ref. 5 and Figs. 1 and 2). It is not clear that all six of the acidic (α) subunits are equally capable of hemagglutinating red blood cells. A relatively lower titer for the globulin would also be expected if the α-β dimers were associated in such a manner as to sterically hinder some of the binding sites on other dimers or perhaps more importantly, if self-binding occurs between the six α-β dimers within the native globulin molecule. Either of these possibilities would result in a lower titer and hence would allow only an apparent binding capability to be measured. Finally, the demonstrated self-interference in the microtiter assay suggests that the carbohydrate attached to the native globulin and the red blood cell carbohydrate compete for the same binding sites. This is consistent with the inhibition of the hemagglutination by the carbohydrate released from the globulin. The occurrence of any self-binding in the more concentrated protein preparation prior to serial dilution would also result in a reduced titer and would permit measurement of only an apparent binding capability.

The failure of all common monosaccharides to inhibit the hemagglutinating activity of this carbohydrate-binding protein is somewhat unusual because the hemagglutinating activity of lectins is frequently inhibited by monosaccharides. Many lectins are known with specificity for polysaccharides (3), with the β-lectin being the most relevant since β-laminarin would inhibit both oat globulin and β-lectins (3). The oat globulin is not a β-lectin, however, even though both lectins are inhibited by β-laminarin. The β-lectins are significantly different in composition for they are glycoproteins composed of 80% carbohydrate and 20% protein (13) in contrast to the oat lectin which is a protein substituted with only 3% carbohydrate. No β-lectins were found when oats were assayed for β-lectin activity (13). The possibility that the hemagglutinating activity was caused by a minor protein contaminant in the cell wall preparation was ruled out by gel electrophoresis analysis (Fig. 3) and by the finding that the specific activity of the material eluted from the affinity column was the same as that of the sample applied to the column (Table I).

The function of the globulin in the cell wall fraction is unknown. The cell walls were washed with a concentration of SDS (1%) sufficient to readily solubilize globulin which may have been associated with them. Thus, it is likely that the globulin extracted from the cell walls was not an artifact of the isolation procedure. Others have observed artifactual binding of lectins to membranes (15), however. We tested the globulin for anti-fungal growth properties against several Ascomycetes which are known to utilize grain as hosts. No effects on fungal growth habits were detected, although this survey was not exhaustive enough to rule out an antifungal role. It is likely that this cell wall fraction globulin is the result of failures in the mechanism storing the globulin and may be of little physiological significance. It is also possible that the globulin-lectin performs some structural function in the cell wall. The discovery of the much more readily solubilized globulin from the cell wall (which was soluble in the absence of salt ["Materials and Methods"]) as compared to the very insoluble globulin from the vacuolar protein bodies of the endosperm (which is only soluble in 1 M NaCl [5]) is fortunate. This very strongly associated, insoluble protein aggregate found in the protein bodies could be the result of carbohydrate-globulin self-binding and the probable protein-protein interactions between globulin molecules. This may be an example in support of Kauss's hypothesis (14) that lectins in plant cells may be strongly bound to their 'receptors' and that the normal extraction procedures probably fail to detach these lectins from their recep-

tors. Associations between lectins and lectin-binding proteins have been reported in seeds of Leguminosa (2, 12). Our observations suggest that solubilization of globulin from within the vacuolar protein bodies was difficult and required the use of severe, denaturing conditions. Furthermore, restoration of the carbohydrate-binding property following these extractions was very difficult to achieve.

The hemagglutinating activity was bound to each of the affinity columns and the activity eluted from each of the columns was inhibited by β-laminarin and by the carbohydrate released from the globulin. The laminarin affinity column bound all of the hemagglutinating activity and less than 5% of the protein applied to the column. All of the hemagglutinating activity was released by treatment with ethylene glycol in buffer. The protein released from this column was identified as globulin by analysis with SDS-polyacrylamide gel electrophoresis (Fig. 3). The specific hemagglutination titer was increased 63-fold to 512 HAU/mg (Table I). This titer is the same as the titer values for globulin prepared from cell walls and for the protein released from the globulin-carbohydrate affinity column. Oat globulin was the major component (>80%) of the sample applied to the globulin-carbohydrate affinity column (Table II). One-half of the total hemagglutinating activity was eluted after treatment with purified carbohydrate released from the globulin. This activity was inhibited by β-laminarin. This evidence is consistent with the identification of the globulin as a lectin with affinity for β-laminarin and for the carbohydrate moieties of the globulin molecule.

The possible self-association of the oat globulin molecules could play an important role in the deposition of the newly synthesized globulin. The globulin is stored in protein bodies (3, 17) within vacuoles (25). Neither the protein bodies nor the vacuoles are enclosed by defined membranes (25). Such a mechanism requires efficient organization of the globulin and self-association by these proteins may be required. The possible capability of globulin molecules to recognize and bind to the carbohydrate of other globulin molecules *in vivo* would provide a high degree of specificity to a self-recognition and self-association mechanism.

An additional and complementary proposal of a role of the possible self-binding *in vivo* includes carbohydrate-globulin binding between the six dimers of the native globulin molecule. This self-binding could participate in the association of the native globulin molecule. It is likely that such associations could occur within the globulin molecule as well as between globulin molecules. If such self-binding occurs *in vivo* its role is probably multifunctional. Similar associations between lectins and lectin-binding proteins have been reported in seeds of Leguminosae (2, 12), but to our knowledge, this is the first report of possible lectin-like binding between subunits of a single protein.

Acknowledgments—We thank Paul Schmidt and Douglas Kelloug for technical assistance. We thank Dr. Robert Bramble for allowing W. G. to conduct some of the experiments in his laboratory.

LITERATURE CITED

1. BARONDES SH 1981 Lectins: their multiple endogenous cellular functions. Annu Rev Biochem 50: 207–231
2. BAUMANN CM, H RÜDIGER 1981 Interactions between the two lectins from *Vicia cracca*. FEBS Lett 136: 279–282
3. BECHTEL DB, Y POMERANZ 1981 Ultrastructure and cytochemistry of mature oat (*Avena sativa* L.) endosperm. The aleurone layer and starchy endosperm. Cereal Chem 58: 61–69
4. BOHLOUL BB, EL SCHMIDT 1974 Lectins: a possible basis for specificity in the rhizobium. Science 185: 269–271
5. BRINEGAR AC, DM PETERSON 1982 Separation and characterization of oat globulin polypeptides. Arch Biochem Biophys 219: 71–79
6. BRINEGAR AC, DM PETERSON 1982 Synthesis of oat globulin precursors. Plant Physiol 70: 1767–1769
7. BROWN JWS, DR ERSLAND, TC HALL 1982 The seed proteins. *In* A Khan, ed, The Physiology and Biochemistry of Seed Development, Dormancy and Germination. Elsevier Biomedical Press, Amsterdam, pp 1–42
8. BURR B, ER BURR, TP ST JOHN, RW DAVIS 1982 Zein storage protein gene family of maize. An assessment of heterogeneity with cloned messenger RNA sequences. J Mol Biol 154: 33–49
9. DUBOIS M, KA GILLES, JK HAMILTON, PA REBEIS, F SMITH 1956 Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356
10. EDGE ASB, CB FALTYNEK, L HOF, LE REICHERT JR, P WEBER 1981 Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal Biochem 118: 131–137
11. GADE W, MA JACK, JB DAHL, EL SCHMIDT, F WOLD 1981 The isolation and characterization of a root lectin from soybean (*Glycine max* (L), cultivar Chippewa). J Biol Chem 256: 12905–12910
12. GANSERA R, H SCHURZ, H RÜDIGER 1979 Lectin-associated proteins from the seeds of Leguminosae. Z. Physiol. Chem. 360: 1579–1585
13. JERMYN MA, YM YEOW 1975 A class of lectins present in the tissue of seed plants. Aust J Plant Physiol 2: 501–531
14. KAUSS H 1977 The possible physiological role of lectins. *In* B Solheim, J Raa, eds, Cell Wall Biochemistry Related to Specificity in Host Plant Pathogen Interactions. Universitetsforlaget, Trums-Oslo-Bergen, pp 347–358
15. KOEHLE H, H KAUSS 1979 Binding of *Ricinus communis* agglutinin to the mitochondrial inner membrane as an artifact during preparation. Biochem J 184: 721–723
16. LIS H, N SHARON 1981 Lectins in higher plants. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 6. Academic Press, New York, pp 371–447
17. LOTT INA 1980 Protein bodies. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 1. Academic Press, New York, pp 589–623
18. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
19. MARES DJ, BA STONE 1973 Studies on wheat endosperm. I. Chemical composition and ultrastructure of the cell walls. Aust J Biol Sci 26: 793–812
20. MIRELMAN D, D GALUN, N SHARON, R LOTAN 1975 Inhibition of fungal growth by wheat germ agglutinin. Nature 256: 414–416
21. MISHKIND ML, BA PALEVITZ, NV RAIKEL 1982 Localization of wheat germ agglutinin-like lectins in various species of gramineae. Science 220: 1290–1292
22. PAULOVA M, M TICHA, G ENTLICHER, JU KOSTIR, J KOCOUREK 1971 Studies on phytohemagglutinins. IX. Metal content and activity of the hemagglutinin from the lentil (*Lens esculenta moench*). Biochim Biophys Acta 252: 388–395
23. PETERSON DM 1978 Subunit structure and composition of oat seed globulin. Plant Physiol 62: 506–509
24. PLANTNER JJ, DM CARLSON 1975 Studies of mucin-type glycoproteins. Anal Biochem 65: 153–163
25. SAIGO RH, DM PETERSON, J HALY 1983 Development of protein bodies in oat starchy endosperm. Can J Bot 61: 1206–1215
26. SPENCER D, TJV HIGGINS 1980 The biosynthesis of legumin in maturing pea seeds. Biochem Int 1: 502–509
27. TREVELYAN WI, DP PROCTER, JS HARRISON 1950 Detection of sugars on paper chromatograms. Nature 166: 444–445
28. TUMER NE, VH THANH, NC NEILSON 1981 Purification and characterization of mRNA precursors. J Biol Chem 256: 8756–8760
29. TURNER RH, IE LIENER 1975 The use of glutaraldehyde-treated erythrocytes for assaying the agglutinating activity of lectins. Anal Biochem 68: 651–653
30. UY R, F WOLD 1977 1,4-Butanediol diglycidyl ether coupling of carbohydrate to Sepharose: affinity adsorbents for lectins and glycosidases. Anal Biochem 81: 98–107